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<p>(21) International Application Number: PCT/EP91/02422 (22) International Filing Date: 16 December 1991 (16.12.91)  (30) Priority data: 9027623.9 20 December 1990 (20.12.90) GB 9105993.1 21 March 1991 (21.03.91) GB  (71) Applicant (for all designated States except US): SMITH-KLINE BEECHAM BIOLOGICALS (S.A.) [BE/BE]; Rue de l'Institut 89, B-1330 Rixensart (BE).  (72) Inventors; and (75) Inventors/Applicants (for US only): VAN WIJNENDALE, Frans [BE/BE]; Teckerstraat 12, B-3040 Ottenburg (BE). BAIJOT, Michelle [BE/BE]; Avenue de Broqueville 153, B-1200 Bruxelles (BE). PRIEELS, Jean-Paul [BE/BE]; Avenue de Fevrier 7, B-1200 Bruxelles (BE).</p>		<p>(74) Agent: DALTON, Marcus, Jonathan, William; Smith-Kline Beecham, Corporate Patents, Great Burgh, Yew Tree Bottom Road, Epsom, Surrey KT18 5XQ (GB).  (81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, KR, LU (European patent), MC (European patent), NL (European patent), SE (European patent), US.  Published With international search report.</p>
<p>(54) Title: VACCINES BASED ON HEPATITIS B SURFACE ANTIGEN  (57) Abstract  Novel antigen are presented which are useful in vaccine formulations for the prophylactic treatment of a range of infectious diseases. The antigens comprise a hybrid polypeptide, one part being the S antigen of the hepatitis B virus, the other being a heterologous antigen, such as gD from HSV. The two antigens are linked by chemical spacers through a native sulphhydryl group present on the surface of the S antigen.</p>		

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## Vaccines based on Hepatitis B surface antigen

The present invention relates to improved immunogens which comprise an antigenic polypeptide chemically conjugated to hepatitis B surface antigen (HBsAg). The invention further relates to novel vaccines and their use.

Proteins or synthetic peptides comprising epitopes of different viruses represent potential immunogens for use in vaccines against the infectious diseases caused by the respective viruses. However, such polypeptides frequently require a combination of carriers and adjuvants to become sufficiently immunogenic for consideration as vaccines.

It would appear that correct antigen presentation is the key requirement for an effective subunit vaccine and Valenzuela et al. (Biotechnology, 1985, 3, 323-326) have concluded that a good immunogen should have the maximum number of its epitopes properly exposed. This requirement was stated by these authors to be difficult to achieve by random chemical coupling of antigens to a carrier molecule. Accordingly a new approach was tried in which HBsAg was used as a carrier and first antigen molecule and the gene encoding a second antigen was recombined with the gene for HBsAg so that the second antigen was assembled into and presented on the surface of the HBsAg particle. Using an N-terminal fusion with a truncated form of the HBsAg middle protein Valenzuela et al. (loc.cit.) observed particle assembly of a Herpes Simplex Glycoprotein D - HBsAg hybrid polypeptide. The glycoprotein D (gD) epitopes were found to be presented in a repetitive fashion at the at the surface of the particle, thereby greatly enhancing the immunogenicity of the gD component.

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More recently it has been shown that the major repetitive epitope of P. falciparum circumsporozoite (CS) protein could be fused to HBsAg. Immunogenicity of the hybrid particles was found to be superior to that of an equivalent monomeric CS antigen (Rutgers et al., Biotechnology, 1988, 6, 1065). Vaccines prepared from hybrid immunogenic particles comprising HBsAg protein are also described in European Patent Application Publication No. 0 278 940. In all cases the hybrid particles were obtained by gene fusion rather than chemical coupling techniques.

Despite the emphasis on gene fusion as a means to designing polyvalent vaccines it has now been found, surprisingly, that effective immunogenic molecules can be prepared by chemical cross-linking of HBsAg protein (or a suitable fragment thereof) having at least one free sulphhydryl group on its surface with another antigen.

Accordingly the present invention provides an immunogenic hybrid polypeptide comprising a first polypeptide component which is HBsAg or fragment thereof displaying the antigenicity of HBV surface antigen, covalently linked via a native sulphur atom in the first polypeptide component to a second polypeptide component.

An advantage of the invention is that an antigen may be coupled to the HBsAg particle with cross-linking agents without impairing the immunogenicity of the HBsAg or fragment thereof as defined hereinabove. Furthermore it is possible by the present invention to conjugate the second polypeptide antigen with a vector (the HBsAg particle) which is able to direct its processing via a non-endosomal route. In this way the second antigen can become associated with MHC I antigens and be recognised as such by cytotoxic lymphocytes. Finally chemical coupling allows a higher degree of freedom with regard to antigen (epitope) density

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on the HBsAg particle and also the possibility of using non-immunogenic spacers whereby the distance from the attachment point to the particle can be varied at will.

5 In one aspect, the hybrid polypeptide of the invention may be represented by formula (I):



10 in which the group  $P^1-S-$  is HBsAg or fragment thereof displaying the antigenicity of HBV surface antigen bonded via a native sulphur atom;

$P^2$  is a second antigenic polypeptide; and

15

X is either:

a) a group  $-A-NH-$  wherein A is a spacer group and  $NH-$  is the residue of an amino group present in the side chain of  
20 an amino acid in  $P^2$ ;

or optionally if  $P^2$  comprises a cysteine residue and a hydrophobic anchor group:

25 b) the sulphur atom of the said cysteine residue present in  $P^2$ .

Preferably  $P_2$  is selected from gD<sub>2</sub>t from HSV or is a peptide capable of binding to HIV neutralising antibodies and  
30 corresponds to the neutralising domain of the  $V_3$  loop from gp120; and

The  $V_3$  loop peptides utilised in the present invention are preferably between 10 to 21 amino acids in length and  
35 comprises a  $\beta$  turn flanked by at least one and preferably at least two amino acid on both sides of the  $\beta$  turn sequences.

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Preferably the sequence corresponds to the sequence 310 to 328 of the gp160 protein.

The following peptide is preferred.

5

TYR THR ARG LYS SER ILE ARG ILE GLN ARG GLY PRO GLY ARG  
ALA PHE VAL THR ILE GLY

The C-terminal tyrosine is optional, as its primary function 10 is to allow labelling with radioactive Iodine.

An important advantage of the hybrid particles according to the present invention is their ability to induce cellular immunity mediated by T lymphocytes and in particular the 15 ability to induce cytotoxic T lymphocytes (CTL) responses.

It will be appreciated that formula (I) is a simplified representation since the second antigen polypeptide  $P^2$  will not normally be bonded to  $P^1$  solely through one sulphur 20 atom. Furthermore,  $P^1$  is preferably in particulate form as described below.

It is essential to the invention that the HBsAg or fragment thereof used as the first polypeptide component has at least 25 one free thiol on its surface (e.g. may be represented as  $P^1$ -SH) and to this end it is important that the HBsAg or fragment thereof is obtained in the correct manner.

Commercially available vaccines against HBV comprise 30 Hepatitis B virus surface antigen (HBsAg) either in native or recombinant form. The authentic Hepatitis B virus surface antigen can be recovered from plasma of infected individuals as a particle of about 22nm comprised of two

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proteins known as P24 and its glycosylated derivative GP28, both of which are encoded by the 226 amino acid coding sequence on the HBV genome known as the S-protein coding sequence or HBV S-gene; see Tiollais et al, Nature, 317 (1985), page 489 and references therein. The complete amino acid sequence of, and nucleotide sequence encoding, HBsAg is given in Valenzuela et al, Nature, 280 (1979), page 815. The numbering system used by Tiollais et al. (loc cit.) to define nucleotide and amino acid positions is used herein.

10

Insertion of HBV S-gene coding sequences under the control of yeast promoters on expression vectors to enable expression of HBsAg in S. cerevisiae for vaccine production has been described by, for example, Harford et al in Devel. Biol. Standard. 54: page 125 (1983), Valenzuela et al., Nature 298, page 347 (1982) and Bitter et al., J. Med. Virol. 25, page 123 (1988). Expression in Pichia pastoris has also been described by Gregg et al, Biotechnology, 5 (1987), page 479 (see also European Patent Application Publication No. 0 226 846) as has expression in Hansenula polymorpha (see EP-A- 0 299 108).

Not all the above methods give HBsAg suitable for use in the present invention since recombinant HBsAg produced in mammalian cells or yeast by the method of Valenzuela and others does not have available free SH groups; it is believed that the cysteine residues of HBsAg are all involved in the formation of disulphide bonds (Wampler et al. (Proc.Natl. Acad. Sci. U.S.A. 1985, 82, 6830-6834 and references therein).

EP-A-0 135435 (assigned to Merck and Co.) describes a method for efficiently converting the non-disulphide bonded HBsAg antigen into a fully intermolecular disulphide bonded particle, alleged to be ten times more immunogenic than the

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antigen which has not been so treated.

However, recombinant HBsAg as produced in S. cerevisiae by SmithKline Beecham Biologicals for the preparation of the vaccine Engerix-B\* (Harford et al. loc.cit.) does have an average of four free cysteines per S monomer and does form particles and this has been found to be at least as immunogenic as the fully intermolecular disulphide bonded particle. It will be apparent that when HBsAg is in this form then free cysteines provide one or more native sulphur atoms which can be utilised for coupling the second polypeptide. It will also be appreciated that preferably the HBsAg forms a particle, typically a lipoprotein particle.

15

It is to be understood that the first polypeptide component in the hybrid according to the present invention may comprise all or part or parts of the HBsAg precursor protein encoded by the coding sequence which immediately precedes the HBV-S gene on the HBV genome referred to herein as the Pre-S coding sequence.

The pre-S coding sequence normally codes for 163 amino acids (in the case the ay HBV sub type) and comprises a pre-S1 coding sequence and a Pre-S2 coding sequence. The latter codes for 55 amino acids and immediately precedes the S-protein coding sequence (see EP-A-0 278 940 for further details).

30 In one preferred aspect the first polypeptide component P<sup>1</sup> is the HBsAg S-protein having one or more, preferably up to 4, sulphhydryl groups on its surface.

\* Engerix-B is a Trade Mark

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From the foregoing it will be appreciated that the first polypeptide component is preferably prepared by recombinant DNA techniques, for example by expression in S. cerevisiae as described by Harford et al (loc.cit.), i.e. corresponds to or comprises the HBsAg antigen present in the commercial vaccine Engerix-B\*.

In another aspect the first polypeptide component may comprise a fragment or truncate of the HBsAg S-protein provided the said fragment has at least one free sulphydryl group on its surface available for coupling to the group X, and provided that particle assembly is not adversely affected.

In yet another aspect the first polypeptide component may be part of a composite particle comprising at least two polypeptides corresponding to part or all of a protein having the biological activity of one of the hepatitis B surface antigens wherein the particle presents at least two antigenic determinants provided by the S-protein, pre-S2-protein or pre-S1-protein, said particle optionally containing host specific lipids, as described in copending European Patent Application No. 0414 374.

An example of such a composite particle may be represented by (L,S) where L is the large protein of HBsAg (including the pre-S1, pre-S2 and S coding sequences as hereinabove defined) and S is the HBsAg S-protein.

In yet a further aspect the first polypeptide component may be a modified L protein of HBsAg as described in copending European Patent Application No. 0414 374 wherein the modified L protein comprises a modified hepatitis B virus large surface protein comprising an amino acid sequence

35

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encoding the L protein characterized by a modification in at least one of the following sequences: a sequence sensitive to protease digestion, a sequence necessary for myristylation, a sequence necessary for N-linked glycosylation, a sequence necessary for O-linked glycosylation, and a sequence necessary for binding of human serum albumin.

In a preferred aspect the modified L protein may be represented by L\* wherein L\* has an amino acid sequence comprising residues 12-52 followed by residues 133-145 followed by residues 175-400 of the L protein.

In another preferred aspect the first polypeptide in the compound of the present invention may be part of a composite particle (L\*,S) wherein L\* and S are as hereinabove defined. Such composite particles may be prepared as described in copending European Patent Application No. 0414 374.

The second polypeptide P<sup>2</sup> is an antigen useful in the preparation of a polyvalent vaccine and may be of any suitable structure.

Specific antigens for P<sup>2</sup> include the recombinant DNA envelope protein gD of Herpes Simplex Virus (HSV), particularly the truncated form of gD known as gD<sub>2t</sub> from HSV2. Other antigens which may be mentioned are malaria antigens, particularly those derived from the circumsporozoite protein, or antigens derived from HIV envelope protein.

The human immunodeficiency virus (HIV) has been identified as the causative pathogen of acquired immunodeficiency syndrome (AIDS). Like other members of the retroviral family, the genes encoding the major structural proteins of the virus

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are defined within the genome by env (viral envelope glycoprotein) and gag (core proteins) genes.

The envelope glycoprotein is known as gp120. After infection with the AIDS virus, human beings develop antibodies against this glycoprotein. In many patients neutralising antibodies are produced although, it is also known that different HIV isolates exhibit a diverse array of sequence variation, particularly in the envelope gene.

10

Peptides from the major neutralising domain located in the envelope protein gp120 in a region known as the V<sub>3</sub> loop are known to bind to neutralising antibodies generated in vivo. Nonetheless in order for those peptides to generate neutralising antibodies, correct presentation of the peptide is required. The present invention achieves this, by presenting such peptides on the surface of HBsAg in the manner described herein.

20 In certain circumstances, the second polypeptide may comprise a hydrophobic anchor group (a hydrophobic 'foot') which may be naturally or synthetically attached to its amino terminal.

25 Suitable hydrophobic anchor groups include fatty acid residues such as myristoyl, palmitoyl and lauryl.

The advantage of having such a hydrophobic anchor group in the second polypeptide component is that it can, via hydrophobic interaction, become embedded in the lipidic membrane associated with the first polypeptide component.

If the second polypeptide component also comprises an accessible cysteine residue the thiol group of the said cysteine can then, by spontaneous oxidation, form an intermolecular disulphide bond with a native thiol group in the first polypeptide component, thereby contributing to the

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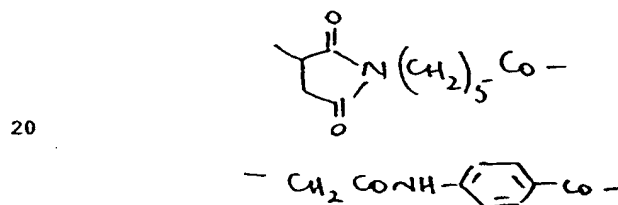
stability of the formed complex. In such a case it is unnecessary to use a chemical cross linking agent to form the hybrid according to the invention (i.e. the group X as hereinabove defined may represent a sulphur atom which is native to P<sup>2</sup> as hereinabove defined).

The linker group A as hereinabove defined represents a linear spacer group bonded at one end to a native sulphur atom of the first polypeptide and at the other end to the second polypeptide P<sup>2</sup> via an amino acid containing side chain in P<sup>2</sup>.

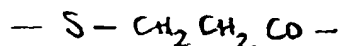
Examples of the group A are substituted C<sub>2</sub>-C<sub>10</sub> alkanes or linear polymers such as polyethylene glycol.

15

Particular groups A include:



and



The present invention further provides a process for the preparation of a hybrid polypeptide of formula (I):



wherein P<sup>1</sup>, P<sup>2</sup> and X are as hereinabove defined which process comprises the steps of

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a) when X is an a group -ANH-:

i) reacting the second polypeptide  $P^2$  with a compound of formula (II):

5



(II)

wherein Y is a group capable of reacting with a native thiol group in the first polypeptide; B is a group which is  
10 specific for one or more amino acid side chains on the second polypeptide  $P^2$  and A is as hereinabove defined; and thereafter

ii) reacting the product with the first polypeptide  
15  $P^1$ -SH; or

b) when X is S and  $P^2$  comprises a hydrophobic anchor group and a cysteine residue:

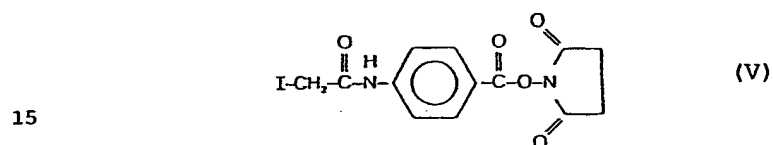
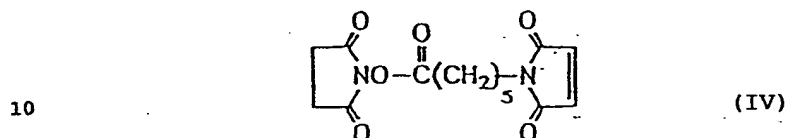
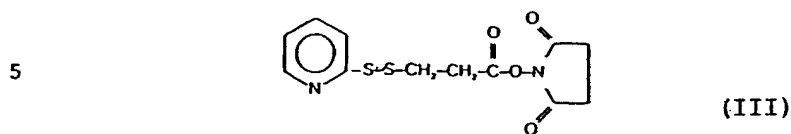
20 i) mixing the first polypeptide and second polypeptide in aqueous solution; and

ii) allowing an intermolecular disulphide bond between  $P^1$  and  $P^2$  to form by spontaneous oxidation.

25

Heterobifunctional reagents of formula (II) are known in the art and include N-succinimidyl 3-(2-pyridylthio)propionate [SPDP; (III)], succinimidyl 6-maleimidyl hexanoate [EMCS (IV)], and N-succinimidyl 4-(iodoacetyl)amino benzoate [SIAB  
30 (V)].

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In method a) according to the above process steps i) and ii) may be carried out standard conditions as known in the art for cross-linking proteins, and the final product may be purified by, for example, preparative high pressure liquid chromatography.

Particular hybrids which are the subject of the present invention include:

25

HBsAg S protein crosslinked to gD<sub>2</sub>t of herpes simplex virus 2 using reagent (V);

HBsAg S protein linked to LCF6 as hereinbelow defined via an intermolecular disulphide bond, and HBsAg S-protein linked to V<sub>3</sub> peptides from HIV gp120;

30

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Typically the ratio of the second polypeptide to the first polypeptide will be in the range 0.1 to 1.0 molecules per monomer. Although in the case of  $V_3$  peptides this may be in the range of 1.0 to 4 molecules per monomer.

5

In a further embodiment of the present invention, the polypeptide  $P^1$  may be mixed with a mixture of  $V_3$  peptides. The resulting fusion with then have different  $V_3$  peptide attached to the surface of  $P^1$ .

10

In the present case the efficiency of the reaction is such that approximately forty peptides are linked to each particle it being understood that the hybrids according to the invention are preferably in particulate form.

15

In a further aspect the invention provides a vaccine composition comprising an immunoprotective amount of a hybrid polypeptide according to the invention together with a conventional carrier or adjuvant.

20

A preferred adjuvant, according to the present invention is de - 3 - 0 - acylated monophosphorylated lipid A (3D-MPL) in a suitable carrier. This adjuvant system provides high neutralising antibody titres.

25

3D-MPL may be obtained by the methods described in U.K. patent No. 2,211502 (RIBI).

In the case of utilising HIV peptides the present inventors  
30 have found that exceptional results may be achieved by first adsorbing the HBsAg- $V_3$  peptide conjugate of the present invention on to alum and then admixing with 3D-MPL.

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Additionally, oil in water emulsions containing 3D-MPL provide excellent results. The oil in water emulsion formulation provided by the present invention most preferably comprises, 3D-MPL, squalane, pluronic L -121 and phosphate buffered saline.

The emulsion is preferably passed through a microfluidizer to provide submicron particles in the emulsion. This enhances the activity of the formulation.

Alternatively vaccines containing the hybrid polypeptide according to the invention are prepared by conventional techniques and will contain an immunoprotective amount of the hybrid preferably in buffered physiological saline and admixed or adsorbed with any of the various known adjuvants including aluminium hydroxide and aluminium phosphate. By "immunoprotective" is meant that enough of the hybrid is administered to elicit a sufficient protective antibody or cell mediated immune response to confer protection against an infectious agent without serious side effects. The amount of hybrid to be administered will depend on whether the vaccine is adjuvanted and will generally comprise between 1 to 1000 mcg of total protein, for example 1 to 200 mcg total protein, more preferably 5 to 40 mcg total protein. The amount and number of doses to be administered can be determined in standard dose range studies involving observation of antibody titres and other responses in subjects.

30

The hybrid polypeptide according to the invention may also be mixed with other antigens such as composite HBsAg particles containing all or part or parts of the PreS1 or PreS2 polypeptides for vaccine formulation. It may also be mixed with fused or other chemically synthesised hybrid

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HBsAg particles carrying epitopes from proteins from other organisms and with other immunogens to form multivalent vaccines. Vaccine preparation is generally described in "Vaccines", edited by Voller et al, University Park Press, 5 Baltimore, MD, U.S.A., 1978.

Accordingly in a further aspect, the invention provides a method of immunising a subject against viral infection which comprises administering to a subject in need of such 10 immunisation an effective amount of a vaccine composition according to the invention.

The following examples illustrate the invention.

#### 15 Examples

##### A. Determination of free sulfhydryls on the recombinant HBsAg particle

#### 20 A.1. Material and Methods

##### a) BNP Method

A method for identifying cysteine-containing peptides in 25 proteins was applied using 2-bromoacetamido-4-nitrophenol (BNP) to introduce an easily detectable probe. The formation of a covalent bond between the protein sulfhydryl group and the acetamido moiety of BNP introduces a chromophore with an absorbance maximum at 410 nm. The 30 modified protein can then be cleaved with appropriate proteases and the resulting peptides separated by chromatographic methods. Monitoring the effluent at a single wavelength (405 nm) provides a rapid and simple method for detecting and isolating only those peptides which 35 contain cysteine residue(s).

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Materials

HBsAg was from SmithKline Biologicals.  $\text{NH}_4\text{HCO}_3$ , 2-bromoacetamido-4-nitrophenol (BNP) and chymotrypsin were 5 from Sigma. Trifluoroacetic acid (TFA) and acetonitrile were from Baker (HPLC grade). N,N-dimethylformamide (DMF) was from Janssen Pharmaceutica.

Protein labelling

10

Protein ( $\pm 1$  mg) was dialyzed against labelling buffer (0.1 M Tris-HCl, EDTA 2 mM, Urea 8M, pH 8.6). 2,5 mg of BNP (dissolved in DMF) per mg of protein was added to the denatured protein. After 90 minutes of incubation at room 15 temperature, unreacted BNP was removed by extensive dialysis against  $\text{NH}_4\text{HCO}_3$  100mM, pH 8.5.

Digestion of modified protein

20 Duplicate addition of chymotrypsin ( $t_0$  and  $t_0 + 4$  hours) (2% W/W) was made. Incubation was then carried out at  $37^\circ\text{C}$  overnight. The digested protein was stored at  $4^\circ\text{C}$ .

HPLC separation of the peptides was accomplished on a Waters 25 600 HPLC system fitted with a 250 mm x 4.6 mm Vydac  $\text{C}_4$  reverse-phase column. The column was equilibrated with HPLC buffer ( $\text{NH}_4\text{HCO}_3$  100 mM, pH 8.5) prior to injection of the sample ( $\pm 1$  mg). Peptides were eluted with a linear acetonitrile gradient from 0 to 50% in 50 minutes. The 30 elution was monitored at 405 nm and at 224 nm on a Waters 490 Multiwave Detector.

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Sequence analysis of the BNP peptides

Dried sample was redissolved in 60 µl TFA 6%, and applied on the glass fiber membrane of the sequenator. The membrane was preferably treated with Biobrene. Edman automatic sequential degradation was performed with a liquid phase sequenator (Applied Biosystem 477A) coupled with an amino acid analyser (Applied Biosystem 120A).

10 The program used is analogous to that of Hewick et al. J. Biol. Chem., 1981, 256, 7990-7997. Phenylthiohyantoin derivatives of cleaved amino acids were identified by RPLC, following the gradient system described by Hunkapiller and Hood, (Methods Enzymol. 1983, 91, 486-493).

15

b) PDS Method

The method of Grasetti et al (Arch. Biochem. Biophys., 1967, 119, 44-49) was followed. Upon reaction of protein thiols with 2,2'-dithiodipyridine (thiol disulfide exchange reaction) 2-thiopyridine is liberated and measured at 343 nm ( $\epsilon_{343 \text{ nm}} = 8.08 \times 10^3 \text{ M}^{-1}$ ).

Materials

25

HBsAg was from SmithKline Biologicals. Dithiothreitol (DTT) and 2,2'-dithiodipyridine (PDS) were from Serva. PD 10 gel filtration columns were from Pharmacia.

30 Assay procedure

Protein ( $\pm 750 \mu\text{g/ml}$ ) was dialyzed against buffer (Urea 8M, EDTA 2 mM, 0.1 M Tris-HCl, pH 8.5). PDS (25 M in excess, dissolved in ethanol) was added to the denatured protein.

35 After one hour of incubation at room temperature, excess of

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PDS was removed by gel filtration (PD 10 column).  
Absorbance of the modified protein was measured at 280 nm.  
Addition of DTT (10 mM final concentration) was made to  
measure the absorbance of the free thiones at 343 nm.

5

c) Carboxymethyl-cysteine determination

Materials

10 Iodoacetamide was from Merck. HBsAg was used from  
SmithKline Biologicals.

Carboxymethylation

15 Protein (1 mg/ml) was dialyzed against buffer (Tris 100 mM,  
EDTA 2mM, pH 8.0) with or without Urea 8M. The  
S-carboxymethylation of the thiol groups was performed by  
addition of iodoacetamide (100 moles per mole of sulfhydryl  
groups) for 20 minutes, in the dark at room temperature.

20

Amino acid analysis

Hydrolysis: Aliquots of protein ( $\pm 100 \mu\text{g}$ ) were dried in  
conic hydrolysis tubes, in a Speed Vac Concentrator.

25 Hydrolysis was performed by addition of HCl 6N (500  $\mu\text{l}$ )  
containing 0.5% phenol, at  $110^{\circ}\text{C}$  during 24 hours. Samples  
were done in triplicate.

After cooling, hydrolysate was evaporated to dryness, washed  
30 in 500  $\mu\text{l}$  water, and dried again. Having been dissolved in  
200  $\mu\text{l}$  of 0.2 N pH 2.2 citrate buffer and filtered on a 45  
 $\mu\text{m}$  membrane, 50  $\mu\text{l}$  of each sample is injected on the  
analyser column.

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Amino acid analysis: was performed with an Automatic Analyser (Alpha Plus-LKB 4151) on a polystyrene sulfonated column. A three buffer elution system was used to separate the different amino acids:

5

1. citrate buffer 0.2 N pH 3.2 at 54°C
2. citrate buffer 0.2 N pH 4.25 at 56°C
3. borate buffer 0.2 N pH 10.0 at 90°C

10 Amino acid detection was made by post-column reaction with ninhydrin and by colorimetric measurement at 440 and 570 nm. For quantification, optical densities at 440 and 570 nm are added together and chromatographic data integration were performed on a Shimadzu CR-A3 integrator.

15

A.2. Results

a) BNP Method

20 After modification of the free thiols by BNP and chymotryptic digestion, peptides containing the BNP label were separated by RPLC and identified by sequencing, following the procedure of Gardner et al. Anal. Biochem., 1987, 677, 140-144.

25

HPLC analysis of 5 different HBsAg chymotryptic digestions yielded 5 well separated major peaks indicating a reproducible oxidation state for all batches. The sequences of the BNP labelled peptides allow the localization of 4  
30 thiol groups (cys 48, 65, 121 and 124).

The identical analysis was performed on HBsAg produced in mammalian cells. No BNP labelled peak could be detected indicating that no free cysteines were available.

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b) PDS Method

Four thiol groups per monomer of HBsAg were detected after application of the PDS method on 3 different batches of HBsAg particles from SmithKline Biologicals (Batches 1 to 3 in Table 1 below) No SH free per monomer could be detected in a batch of HBsAg expressed in Chinese hamster ovary (CHO) cells (Batch 4).

10 Table 1:

PDS Method: FREE THIOL GROUPS DETERMINATION

15	BATCH NUMBER	SH FREE PER MONOMER
20	1 (yeast) 2 (yeast) 3 (yeast) 4 (CHO cells)	4.38 3.18 4.2 0

25 c) Carboxymethyl-cysteine determination

Cysteines in HBsAg particles were carboxymethylated with or without denaturing agent (Urea 8M).

30 Subsequent amino acid analysis showed the presence of 3 carboxymethylcysteine per monomer of HBsAg in both cases.

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B. Examples of coupling antigens to the HBsAg particleB.1. EXAMPLE 1: Covalent coupling of the glycoprotein D of Herpes Simplex 2 Virus to a particulate carrier

5

B.1.1. Introduction

The glycoprotein D of HSV 2 (gD<sub>2</sub>t) expressed in CHO cells (Lasky and Dowbenko DNA, 1984, 3(1), 23-29) is covalently  
10 coupled to a recombinant HBsAg particle containing free SH groups.

B.1.2. Materials and Methods15 a. Agents

. 5,5'-Dithiobis 2-nitrobenzoic acid (Ellman's reagent or DTNB) and N-succinimidyl(4-iodoacetyl)-aminobenzoate (SIAB) were purchased from PIERCE.

20

. 2,4,6-Trinitrobenzenesulfonic acid (TNBS) was obtained from SERVA.

. Recombinant gD<sub>2</sub>t was expressed in CHO cells and  
25 purified by SmithKline Biologicals.

. gD<sub>2</sub>t was iodinated by the enzymobeads method of PIERCE.

30 . HBsAg particles were produced by SmithKline Biologicals.

b. Methods

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b.1. Characterization of gD<sub>2</sub>t

## b.1.1 Quantitative determination of sulphhydryl groups:

5 To 150 µl of gD<sub>2</sub>t (23 µM in Na<sub>2</sub>HPO<sub>4</sub> 0.02 M pH 7) 1 ml of DTNB (3.28 mM in Na<sub>2</sub>HPO<sub>4</sub> 0.04 M pH 8) is added.

After 5 min, the optical density at 412 nm is determined against a blank lacking protein. An extinction coefficient  
10 of  $1.3 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$  is used to calculate the concentration of sulphhydryl groups reacting (Ellman, Arch. Biochem. Biophys., 1959, 82, 70).

## b.1.2 Quantitative determination of lysine residues:

15

50 µl of TNBS/H<sub>2</sub>O 24.5 mM are added to 50 µl of gD<sub>2</sub>t (58 µM in Na<sub>2</sub>HPO<sub>4</sub> 0.02 M pH 7) diluted in 200 µl of borate buffer (0.05 M Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub> adjusted to pH 9.5 with 0.05 M NaOH).

20 After 3 hours in the dark at room temperature the change in absorbance at 367 nm is followed against a blank without protein.

The extent of trinitrophenylation is calculated on the basis  
25 of an  $\epsilon_{367 \text{ nm}} = 1.1 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$  (Plapp et al., J. Biol. Chem. 1971, 246 (4), 939-945).

b.2. Activation of gD<sub>2</sub>t with SIAB

30 100 µl of gD<sub>2</sub>t (23 µM in Na<sub>2</sub>HPO<sub>4</sub> 0.02 M pH 7) + 100 µl gD<sub>2</sub>t I<sup>125</sup> are incubated for 30 min at 37°C with 2 µl SIAB (25 mM in DMSO) which corresponds to a molar ratio succinimide/lysine of 2. The excess of cross-linker is eliminated by dialysis (2 hours against Na<sub>2</sub>HPO<sub>4</sub> 0.02 M pH 8)  
35 and the reaction mixture is concentrated to 100 µl by

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ultrafiltration on an YM 10 centricon.

b.3. Coupling to HBsAg particle

5 gD<sub>2</sub>t (100 µl), concentrated (1 mg/ml) and SIAB-activated, is incubated with 53 µl of HBsAg particles (1 mg/ml in Na<sub>2</sub>HPO<sub>4</sub> 10 mM pH 7.2, NaCl 150 mM) for various times at 37°C.

The initial molar ratio gD<sub>2</sub>/S monomer is 1/1.

10 The particulate gD<sub>2</sub>t is purified by a 1.5 M CsCl gradient (45 hours, 65000 rpm in a 70.1 Ti rotor).

b.4. Quantification of gD<sub>2</sub>t coupled per particle

15 50 µl of water are added to a vial of enzymobeads. After one hour, 50 µl of Na<sub>2</sub>HPO<sub>4</sub> 0.2 M pH 7.2, 25 µl gD<sub>2</sub>t (1 mg/ml), 0.5 mCi NaI<sup>125</sup> (Amersham) and 25 µl 1% β-D-Glucose are added.

20 After 20 min at room temperature, the reaction is completed and the iodinated protein is separated from free iodine by chromatography on DOWEX Ag 1 x 8 resin saturated by BSA 1%.

The specific activity of the gD<sub>2</sub>t involved in the coupling  
25 may be determined by the radioactivity detected in the mixture of labelled and non-labelled gD<sub>2</sub>t. The amount of gD<sub>2</sub>t coupled to particles may be determined by this specific activity.

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### B.1.3. Results

#### a. Characterization of gD<sub>2</sub>t

5 a.1. Quantitative determination of sulfhydryl groups No  
free thiol is detected on the gD<sub>2</sub>t by DTNB. This result  
fits the aminoacid sequence of the protein. The truncated  
gD<sub>2</sub>t molecule used (283 aa) contains 6 cysteine residues,  
each involved in disulfide bridges that constitute  
10 discontinuous epitopes. Therefore, gD<sub>2</sub>t is an ideal  
molecule for the activation step with a heterobifunctional  
cross-linker without risk of homopolymerisation.

#### a.2. Quantitative determination of lysine residues

15

The number of free amino groups are detected by TNBS either  
on the native or on the SIAB activated gD<sub>2</sub>t. The number of  
free lysines decreases as a function of the excess of SIAB.

20 With a molar ratio SIAB/lysine of 2, four residues are  
activated.

The number of detected lysines on the native protein (10) is  
close to the number determined in the amino acid sequence  
25 (11).

#### b. Activation of gD<sub>2</sub>t by SIAB

After an activation of 30 min at 37°C with a molar ratio of  
30 SIAB/lysine of 2, the absence of homopolymers of gD<sub>2</sub>t is  
checked by gel filtration.

On a TSK 3000 column, the homopolymers elute in the void  
volume (8 min) and the monomeric gD<sub>2</sub>t has a retention time  
35 of 15 min.

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Despite the absence of cysteine residues in the protein, formation of homopolymers is observed when gD<sub>2</sub>t is activated at a concentration of 2.5 mg/ml. An aspecific reactivity of the halogen in SIAB for lysine, methionine or histidine 5 residues may explain this phenomenon (see Means and Feeney, Chemical Modifications of Proteins; Holden Day publ., 1971, page 107).

Initial protein concentration is a decisive factor in 10 homopolymerisation events. If gD<sub>2</sub>t is activated at a concentration of 0.5 mg/ml, the formation of homopolymers decreases from 50 to 10%.

c. Coupling to HBsAg particle

15

The HBsAg-gD<sub>2</sub>t conjugate obtained after a 30 min, 2 hrs or over night incubation at 37°C is purified by CsCl gradient. The gD<sub>2</sub>t homopolymers have a different density to the carrier and do not contaminate the conjugate. The yield of 20 coupling increases with time as shown in Table 2. 0.2 gD<sub>2</sub>t molecules are coupled per S monomer (twenty per particle) as calculated by radioactivity detected in the particle's density area.

25 Table 2: Influence of incubation on the yield of coupling

	Time	gD <sub>2</sub> t/S monomer
30	30 min	0.08
	2 hrs	0.14
	15 hrs	0.20

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B.2. Example 2B.2.1. Incorporation of LCF6 [Lauroyl-Cys-Tyr-Gly-Gly-(NPNA)6] into HBsAg particles

5

10 µl of LCF6 solution (1 mg/ml in phosphate 10 mM pH 7, NaCl 150 mM) are incubated with 10 µl of a solution of yeast expressed HBsAg particle (ex SmithKline Biologicals) at 1 mg/ml in phosphate 10 mM pH 7, NaCl 150 mM overnight at 10 37°C.

B.2.2. Assay

Quantitation of the HBsAg-LCF6 hybrid is performed by a 15 sandwich ELISA test using as coating antibody a polyclonal IgG against HBsAg at a concentration of 5 µl/ml and as detecting antibody a biotinylated monoclonal IgM directed against the repeat sequence of LCF6.

20 An antigen R16 HBsAg solution (a recombinant repeat Malaria-HBsAg particle) of known concentration is used as standard. This ELISA presents the advantage of monitoring the hybrid without prior separation of the free peptides. The results are expressed in µg/ml equivalent R16 HBsAg.

25

B.2.3. Implication of a covalent bond in the LCF6 incorporation into HBsAg particle

To evaluate the relative important of the disulfide bridge 30 and of the hydrophobic foot, the incorporation of the peptide without cysteine and of the peptide without the lauroyl group is compared. The ELISA shows that the lipopeptide lacking cysteine (LF6) fails to incorporate into the HBsAg particle. The peptide without the lauroyl group 35 (CF6) is nearly ineffective. Therefore the synergy between

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hydrophobic and covalent interactions is critical for the peptide's incorporation into HBsAg particle.

The results from LCF6 incorporation into different HBsAg particles with variable oxidation state were compared.

The lipopeptide is incubated with classical HBsAg particle ( $\pm$  4 free SH groups/S monomer), with more oxidized particles (1 free SH group/S monomer), or with totally oxidized particles (no free SH group; particles synthesized in CHO cells).

These results indicated that a disulfide bridge between LCF6 and S monomer stabilizes the peptide incorporation. The ELISA for anti-HBsAg-LCF6 show decreasing coupling as a function of an increasing oxidation state of HBsAg particles.

The existence of covalent linkage between LCF6 and HBsAg particle is also demonstrated by analysis on SDS-PAGE. Bands at 26 and 50 Kd appear in non-reducing conditions. These bands correspond to the LCF6 peptide coupled to the S monomer and dimer.

In contrast, in reducing conditions, these bands disappear.

B3. Example 3 : Incorporation of V<sub>3</sub> peptides into HBsAg particles

3.1. V<sub>3</sub> loop structure

The peptide used represents the sequence from amino acid 310 to 328 (G. Larosa, Science 1990, 249-932) comprised in a disulfide bridged loop in the third variable region of the external protein gp120. Its conservation is over 80% in 9

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out of 14 positions in the central portion and its predicted structural motif is of the type  $\beta$  strand-type II  $\beta$  turn- $\beta$  strand- $\alpha$  helix.

5 The primary structure of the peptide can be represented by the following

TYR THR ARG LYS SER ILE ARG ILE GLN ARG GLY PRO GLY ARG  
ALA PHE VAL THR ILE GLY

10

### 3.2. Synthesis

The peptide is synthesized according to the Merrifield solid phase method, giving, after purification by reverse phase 15 hplc, a peptide of 97% purity. The peptide is homogeneous by SDS-PAGE and gel filtration on TSK 2000 column. Its molecular weight is 2800.

### 20 3.3. Coupling strategy

EMCS, (succinimidyl 6-maleimidyl hexanoate an heterofunctional crosslinker containing a primary amino reactive group N-hydroxysuccinimide (NHS) and a thiol group 25 (maleimide), was chosen.

#### 3.3.1. Activation

The V<sub>3</sub> peptide contains one Lysine group which was activated 30 with EMCS for 30 min at 37°C, pH 7, in a 1:1 to 4:1 ratio.

#### 3.3.2. Coupling to HBs

After elimination of the excess of crosslinker on a G 10 35 column, HBsAg particles were added in a 1:1 ratio with the

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V<sub>3</sub> peptide. The reaction took place overnight at 37°C, pH 6.5 - 7.5.

The conjugate obtained is purified from the unconjugated peptide by gel filtration on HR 200 column.

### 3.3.3. Coupling efficiency

As assayed by radioimmunoassay and ELISA, the coupling efficiency was of 0.4 peptide coupled per HBs monomer, which is equivalent to 40 V<sub>3</sub> peptides per HBs particle or to 1 mg V<sub>3</sub> per 25 mg HBsAg.

## 15 Example C

### Antigen-Adjuvant preparations

#### 1. V<sub>3</sub> HBsAg particles in oil water emulsion

20

The vehicle is prepared as follows. To phosphate-buffered saline (PBS) containing 0.4% (v/v) Tween 80 is added 5% (v/v) Pluronic L121 and 10% squalane. This mixture is then microfluidized. For microfluidization, the emulsion is  
25 cycled ten times through a microfluidizer (Model M110 Microfluidics Corp., Newton, Mass.). After five passes through the Microfluidizer, the resulting emulsion comprises only submicron particles. 50 µg 3D-MPL is then added to this emulsion. One volume of this emulsion containing 3D-MPL  
30 is mixed with an equal volume of twice concentrated V<sub>3</sub> HBsAg and vortex briefly to ensure complete mixing of the components. The final preparation consists of 0.2% Tween 80, 2.5% Pluronic L121, 5% squalane, 50 µg 3D-MPL and 1 µg equivalent V<sub>3</sub> peptide (corresponding to 25 µg HBsAg) in a  
35 250 µl injection dose.

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1.2 V<sub>3</sub> HBsAg/Aluminium Hydroxyde plus 3D-MPL

1 µg equivalent V<sub>3</sub> is adsorbed overnight at 4°C on alum corresponding to 0.5 mg equivalents Al<sup>3+</sup> in 0.25 ml of 150 mM NaCl, 10 mM phosphate buffer pH 6.8. After overnight incubation, the adjuvant preparation is centrifuged and its supernatant removed. An equal volume of adsorption buffer containing 50 µg 3D-MPL is then added to the alum-bound HBsAg - V<sub>3</sub> peptide.

10

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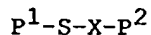


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VACCINESClaims

5 1. An immunogenic hybrid polypeptide comprising a first polypeptide component ( $P^1$ ) which is HBsAg or a fragment thereof displaying the antigenicity of HBV surface antigen, covalently linked via a native sulphur atom in the first polypeptide component to a second polypeptide component  
10 ( $P^2$ ).

2. A hybrid polypeptide of the formula



15

in which  $P^1-S-$  is HBsAg or fragment thereof displaying the antigenicity of HBV surface antigen bonded via a native sulphur atom;

20

$P^2$  is a second antigenic polypeptide; and

X is either:

25 a) a group  $-A-NH-$  wherein A is a spacer group and  $NH-$  is the residue of an amino group present in the side chain of an amino acid in  $P^2$ ;

or optionally if  $P^2$  comprises a cysteine residue and a  
30 hydrophobic anchor group:

b) the sulphur atom of the cysteine residue in  $P^2$ .

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3. A hybrid polypeptide as claimed in claim 1 or 2, wherein  $P^2$  is selected from, gD of Herpes Simplex Virus or a derivative thereof, gp120 from HIV or a derivative thereof, the circumsporozoite antigen or derivative thereof.

5

4. A hybrid polypeptide as claimed in claim 3 wherein  $P^2$  is gD<sub>2t</sub>.

5. A hybrid polypeptide as claimed in claim 3 wherein  $P^2$  is a peptide corresponding to the  $V_3$  loop of HIV gp120.

6. A hybrid polypeptide as claimed in any of claims 1 to 5 wherein  $P^1$  comprises all or part or parts of the HBs Ag precursor protein.

15

7. A hybrid polypeptide as claimed in any of claims 1 to 6 wherein  $P^1$  is monomer of the particle.

8. A hybrid protein as claimed herein, wherein before fusion  $P^1$  is HBsAg-S-protein having one or more sulphydryl groups on its surface.

9. A hybrid protein as claimed herein wherein  $P^1$  is a part of a composite particle comprising at least two polypeptides corresponding to part or all of a protein having the biological activity of the hepatitis B surface antigen wherein the particle presents at least two antigenic determinants provided by the S-protein, pre S2-protein or pre S1-protein, said particle optionally containing host specific lipids.

10. A hybrid protein as claimed in claim 9 wherein the composite particle maybe represented by L, S, wherein L is the large protein of HBsAg, including pre S1, pre S2 and S, and S is HBsAg S-protein.

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11. A hybrid protein as claimed in claim 10, characterised is that the L protein comprises one or more of the following modifications in the following sequences:

- 5 a sequence sensitive to protease digestion,
- a sequence necessary for myristylation,
- a sequence necessary for N-linked glycosylation,
- a sequence necessary for O-linked glycosylation, and
- a sequence necessary for binding of human serum albumin.

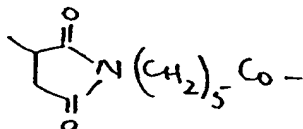
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12. A hybrid protein as claimed in claim 10 or 11, wherein L is modified and has an amino acid sequence comprising residues 12-52, followed by 133-145, followed by residues 175-400 of the L-protein.

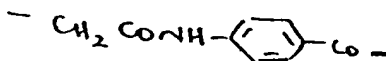
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13. A hybrid protein as claimed in any of claims 2 to 12 wherein A is a substituted  $C_2-C_{10}$  alkane or a linear polymer such as polyethylene glycol or a compound

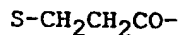
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25



and



14. A vaccine formulation comprising a hybrid protein as  
30 claimed in any of claims 1 to 13 in conjunction with a pharmaceutically acceptable excipient.

15. A vaccine formulation as claimed in claim 14 further comprising de - 3 - O - acylated monophosphorylated lipid A  
35 (3D-MPL) in a suitable carrier.

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16. A vaccine formulation as claimed herein comprising 3D-MPL and alum.

17. A vaccine formulation as claimed herein wherein the carrier is an oil in water emulsion.

18. A vaccine formulation as claimed in claim 18 wherein the oil in water emulsion comprising 3D-MPL, squalane, pluronic L - 121 and phosphate buffered saline.

10

19. A process for the preparation of a hybrid polypeptide of formula (I):



15

wherein  $P^1$ ,  $P^2$  and X are as defined in claim 2 which process comprises the steps of

a) when X is an a group -ANH-:

20

i) reacting the second polypeptide  $P^2$  with a compound of formula (II):



25

wherein Y is a group capable of reacting with a native thiol group in the first polypeptide; B is a group which is specific for one or more amino acid side chains on the second polypeptide  $P^2$  and A is as hereinabove defined; and

30 thereafter

ii) reacting the product with the first polypeptide  $P^1$ -SH; or

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b) when X is S and P<sup>2</sup> comprises a hydrophobic anchor group and a cysteine residue:

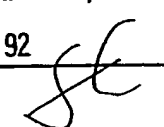
- i) mixing the first polypeptide and second polypeptide  
5 in aqueous solution; and
- ii) allowing an intermolecular disulphide bond between P<sup>1</sup>  
and P<sup>2</sup> to form by spontaneous oxidation.

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## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 91/02422

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 C07K15/04; A61K39/29		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
Int.Cl. 5	C07K ; A61K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
A	EP,A,0 271 302 (SCRIPPS CLINIC AND RESEARCH FOUNDATION) 15 June 1988 see page 3, line 10 - line 30 see page 6, line 55 - page 7, line 33 see page 7, line 48 - line 64 see page 11, line 9 - line 21 see page 12, line 54 - page 13, line 25	1-19
A	EP,A,0 326 109 (NEW YORK BLOOD CENTER) 2 August 1989 see page 2, line 28 - page 3, line 37 see page 5, line 35 - page 8, line 15	1-19
A	EP,A,0 385 610 (THE WELLCOME FOUNDATION) 5 September 1990 see page 2, line 38 - page 3, line 20	1-19
-/-		
<p><sup>10</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"A" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
16 MARCH 1992	19. 03. 92	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	KORSNER S.E. 	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	<p>PROCEEDINGS OF THE AMERICAN NATIONAL ACADEMY OF SCIENCE vol. 85, 1988, WASHINGTON pages 1932 - 1936; PALKER ET AL: 'Type-specific neutralization of the human immunodeficiency virus with antibodies to ENV-coded synthetic peptides' * P. 1932 ( Abstract ) * * P. 1933 ( Col. 1, top ) *</p>	1-19
A	<p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCE U.S.A. vol. 85, 1988, WASHINGTON pages 4478 - 4482; GOUDSMIT ET AL: 'Human immunodeficiency virus type I neutralization epitope with conserved architecture elicits early type-specific antibodies in experimentally infected chimpanzees' * P. 4478 ( Abstract ) *</p>	1-19
A	<p>JOURNAL OF PROTEIN CHEMISTRY vol. 2, no. 3, 1983, NEW YORK pages 263 - 277; PARTIS ET AL: 'Cross-linking of protein by W-maleimido alkanoyl N-hydroxysuccinimide esters' * P. 267-270 *</p>	1-19
A	<p>BIOTECHNOLOGY vol. 3, no. 4, 1985, LONDON pages 323 - 326; VALENZUELA ET AL: 'Antigen engineering in yeast: Synthesis and assembly of hybrid hepatitis B surface antigen-herpes simplex 1 gD particles' * P. 323 *</p>	1-19

**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO. EP 9102422  
SA 54130**

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
The members are as contained in the European Patent Office EDP file on  
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		US-A- 4818527	04-04-89
		AU-A- 8223187	09-06-88
		JP-A- 1025800	27-01-89
EP-A-0326109	02-08-89	US-A- 5039522	13-08-91
EP-A-0385610	05-09-90	AU-A- 4975590	18-10-90
		JP-A- 3027400	05-02-91

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